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Increase of Peroxidase Activity Unrelated with Resistance to Rice Blast Disease

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松山宣明*・高坂淳爾**：イネいもち病抵抗性と相関しない Peroxidase の活性増について

Abstract

In an incompatible combination of rice cultivar and blast fungus race, the rapid increase of peroxidase activity of rice leaves occured within 24 hr after inoculation, while a little or no increase was observed in a compatible case at early stage of infection. However, sudden increase occured at 3-5 days after inoculation in a compatible combination and the activity exceeded that of an incompatible combination. These alteration of activities occured more rapidly in aged leaves. The highest activity was observed at the time of rapid lesion enlargement in compatible case. The isozymes activated by the infection were fixed, and no new isozyme specific to compatible or incompatible combination was detected. The distinct difference between both combinations was the time of increase started. The ample supplies of nitrogenous fertilizer reduced the degree of resistance to rice blast disease. However, peroxidase activities increased with the doses of N-fertilizer and the highest activity was observed at the highest dose, i.e., under the most susceptible condition. These results indicate that the increase of peroxidase activity is possibly the reflection of host cell collapse or physiological disorder and may not be directly involved in the resistance mechanism.

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Introduction

The shift of oxidative enzyme activities reflects a rapid physiological response of host cells in infection site and is commonly observed in various plant diseases. The increased activity of peroxidase after infection have been well documented[5,18,37,41,48~50]. Although the role and site of action of peroxidase are still in ambiguity, some close relations with physiological processes have been clarified. Peroxidase plays as a catalyst of ethylene,[47] lignin[13,33,34] and phytoalexin[4] formation, further it may, in some cases, participate in the oxidation of phenolics[20,54] and direct inhibition of parasite's growth[23] through such as myeloperoxidase-halide-hydrogen peroxide antibacterial system[19]. Therefore, the implication of peroxidase in resistance to various plant diseases have been much stressed. Some reports indicated that the qualitative and quantitative changes of peroxidase isozymes may associate with determining resistance or susceptibility[12,22,45,51]. However, in some cases it was indicated that the increase of peroxidase activities or appearance of new isozymes occured as a result of symptom response, or may be related with the degeneration of host cells caused by infection[11,21,29]. The critical reports describing the increase of peroxidase activities unrelated with the resistance have been

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recently accumulated[1,3,6~10,14~17,24,31,36,38,40,42,52,53]. These results, however, were mainly on unrelated increase of activity with race-specific resistance and not with the general resistance which changes according to the ageing of leaf tissues or various environmental conditions. In this report the alteration of total and individual isozyme activity of peroxidase in compatible and incompatible combinations in rice blast disease caused by *Pyricularia oryzae* Cav. was compared and, furthermore, the increase of activity which does not correlate with the general resistance in compatible combination will also be reported. The abstracts and similar data have partly been reported elesewhere[25~28]. Most experiments were carried out at National Institute of Agricultural Sciences, Tokyo.

Materials and Methods

Isolates. The isolates of *Pyricularia oryzae* Cav. used in this experiment were Ken 68–17 (NIAS) and TH 65–105 (Tohoku National Agricultural Experiment Station).

Exp. 1. Rice cultivar Kanto 51 (*Pi-k*) which is susceptible to Ken 68–17 and resistant to TH 65–105 was sown in soil mixture of Nishigahara soil and paddy field soil (6 : 4) fertilized with 1 g of pre-mixed fertilizer (Chiyoda Kasei Co. Ltd.) in plastic pots (15×6×10 cm). At 5–leaf stage, spray inoculation was routinely carried out with a spore suspension (ca. 10 spores under ×150 microscopic field). Plants sprayed were incubated in a moist chamber at 24 C for 17 hr. Then, inoculated plants were transferred to a green house air-conditioned around 27 C. Sampling of 4 th and 5 th leaves was carried out at 0, 1, 3, 5 and 7 th day after inoculation. Samples collected were wrapped with aluminium foil and stored at −10 C until the final sampling at 7 th day. The preparation of peroxidase from every sample was carried out at the same day. Time courses of total and isozyme activities of peroxidase after infection in compatible and incompatible combinations of host and parasite were compared.

Exp. 2. Cultivar Aichi Asahi (*Pi-a*) was preliminarily raised on the mesh-screen floated on tap water which was adjusted to pH 5.0–5.5. At 2–leaf stage, seedlings of uniform size were selected and transplanted to vermiculite in 15 cm porcelain pots, six seedlings per each pot. One-fourth strength of Kasugai's culture solution[2] was watered every 4 days for 8 days after transplanting and followed by 1/2 strength solution. When 6 th leaves developed fully, the ammonium sulfate solutions containing 188 and 376 mg of $(NH_4)_2SO_4$ respectively were added to some pots. These treatments were designated as Tr–1 and Tr–2 respectively. The ample supplies of ammonium sulfate was repeated once more at 5 th day after the first treatment. When 7 th leaves developed fully, 6 th and 7 th leaves were sampled separately. The alteration of peroxidase activity in amply N-fertilized rice plant which reduced its resistance to rice blast was examined.

Preparation of peroxidase. In Exp. 1, 1 g of leaf sample was homogenized in 5 ml of 12.5 % glucose containing 1/15M phosphate buffer (pH 7.4) with the addition of quartz sand in a mortar. The homogenate was centrifuged at 12,000×*g* for 10 min. Then, 2 ml of cold acetone was added to 1 ml of the supernatant in a small centrifuge tube and centrifuged at 6,000×*g* for 10 min. Resulting precipitate was resuspended to 3 ml of cold acetone and centrifuged as stated above. The washing was repeated once more and remaining acetone was completely eliminated *in vacuo*. The precipitate was transferred to a Tefron homogenizer with distilled water, homogenized slowly to make

a emulsion in ice cold. The homogenate was finally adjusted to 60 ml with distilled water and used as the enzyme solution. The supernatants before acetone treatment were used as the enzyme preparations for gel electrophoresis. In Exp. 2, the preparation of enzyme was conducted as stated above except for using 1/60M phosphate buffer (pH 7.4) and addition of equal volume of cold acetone for precipitation of enzyme. All procedures were carried out volumetrically in the cold.

Peroxidase activity. Five ml of 0.2M acetate buffer (pH 4.7), 1 ml of 1% *o*-phenylenediamine ethanol solution, 1 ml of 0.3% H_2O_2 solution and 200 μl of the enzyme solution were incubated at 30 C for 5 min. Then, 1 ml of saturated sodium bisulfite solution was added to stop the reaction. The activity was measured colorimetrically by O. D. at 430 nm with a spectrophotometer (Beckman Co.).

Thin layer electrophoresis. The enzyme preparations were subjected to polyacrylamide gel thin layer electrophoresis. Nitto GE (Acrylamide 92% and ethyleneureabisacrylamide 8%) was used as a supporting medium with DMAPN (Dimethylamino propionitrile) and ammonium persulfate as catalysts. A 4% gel was used with discontinuous buffer system (Gel buffer : 0.038M Tris hydroxymethylaminomethane–0.05 M NaOH, pH 8.2). Ten μl of each sample was poured into gel slots. The samples from compatible and incompatible combinations were always run on a same gel sheet with the check sample from healthy leaves. The running was conducted at ca. 5 C for about 100 min at constant 1 mA/cm (width of the gel).

Detection and densitometry. *o*-Dianisidine which is the most stable electron donor and was recommended for the detection of peroxidase[35] was used. One hundred mg of *o*-dianisidine was dissolved in 50 ml of 95% ethanol and mixed with 49 ml of 0.2M acetate buffer (pH 4.7). One ml of 0.3% H_2O_2 (final concentration is ca. 0.003%) was added to the mixture stated above and poured in the tray. Gel was dipped in and the tray was floated on 30 C water bath. Fifteen min after dipping, 1 ml of saturated sodium bisulfite was added to stop the reaction. The gel was immediately transferred to Chromoscan densitometer (Joyce Loebl Co.) and the activity of each isozyme was measured colorimetrically by O. D. at 430 nm.

Results

The alterations of peroxidase activities after inoculation were compared in compatible and incompatible combinations of rice cultivar and races of *Pyricularia oryzae* Cav. In the incompatible combination, the total activities at 5 th leaves increased within 24 hr after inoculation and successively increased. While in the compatible combination, the activity was stable until 3 rd day and followed by rapid increase, reaching its maximum at 5 th day and then slightly decreased toward 7 th day. The activities in the compatible case at 5–7 th day were higher than that of the incompatible case (Fig. 1–a). The highest increase was observed at the time of rapid enlargement of blast lesions in the compatible combination. The increase of activities occured more rapidly at 4 th leaves (second leaf from the top) (Fig. 1–b). The gradual increase was observed in intact check leaves.

Although total activity is the reflection of each isozyme activity, total activities were affected by activities of the major isozymes. Isozyme a, b, d and g were mainly increased

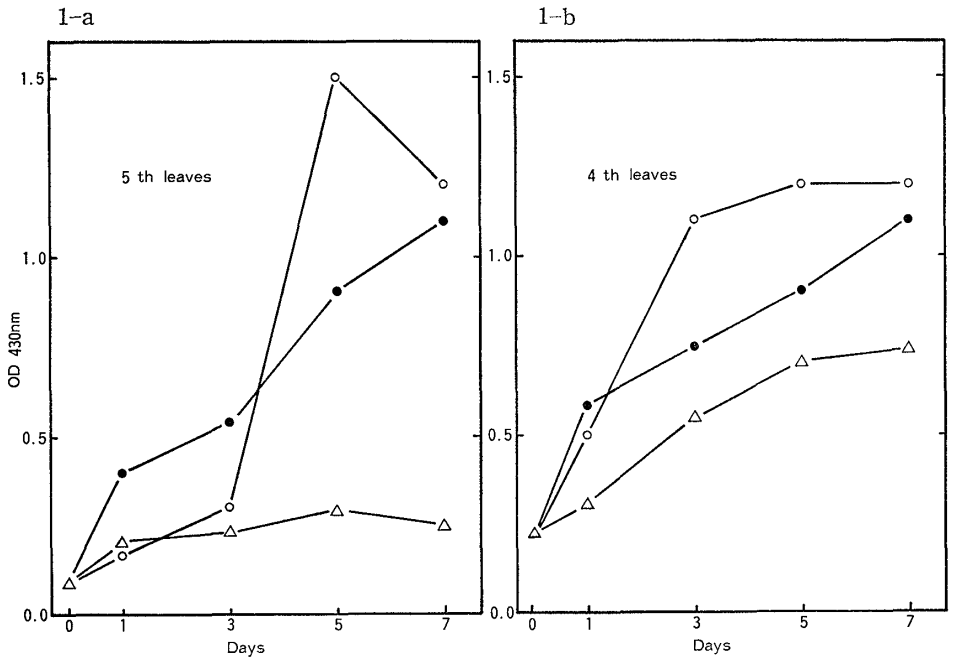

Fig. 1. Time course of peroxidase activities of 5 th (top) leaves (1-a) and 4 th (second) leaves (1-b) inoculated with compatible and incompatible isolates.
(○): Compatible, (●): Incompatible, (△): Check
Cultivar: Kanto 51, Inocula: Ken 68-17 (Compatible), TH 65-105 (Incompatible)

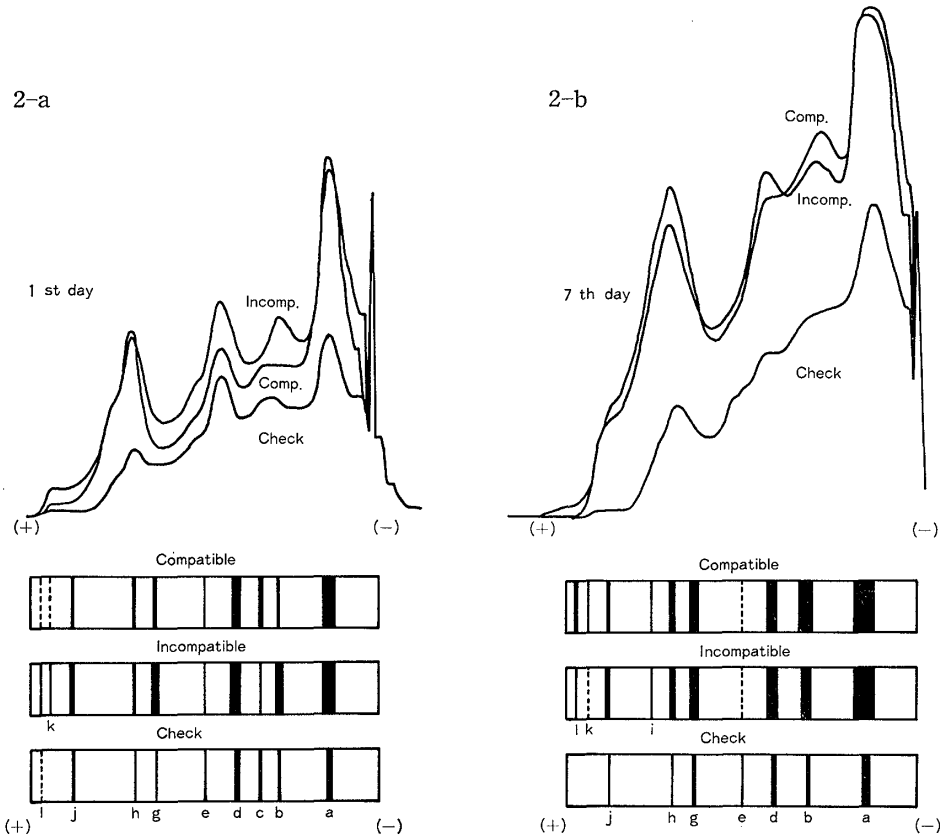

Fig. 2. Densitometry and diagrammatic representation of peroxidase zymograms of 5 th (top) leaves inoculated with compatible (Comp.) and incompatible (Incomp.) isolates at 1 st (2-a) and 7 th (2-b) day after inoculation. Thin layer polyacrylamide gel electrophoresis was carried out and three samples were run on the same gel sheet and activities were compared under same condition. Cultivar: Kanto 51, Inocula: Ken 68-17 (Compatible), TH 65-105 (Incompatible)

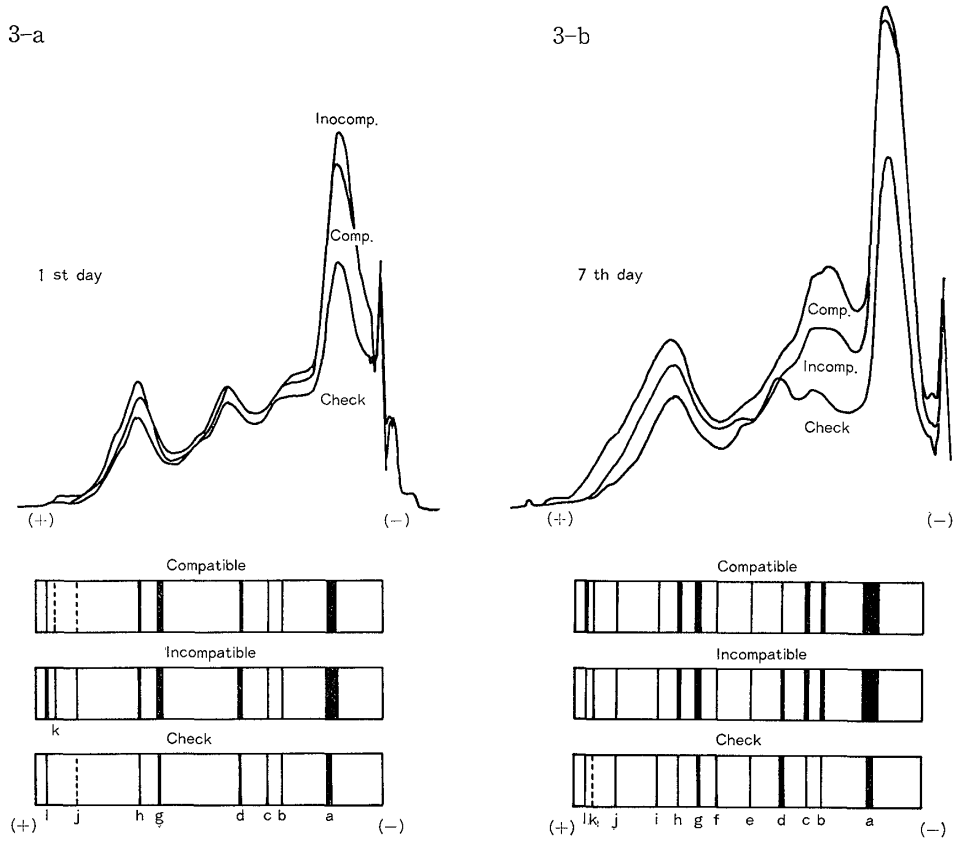

Fig. 3. Densitometry and diagrammatic representation of peroxidase zymograms of 4th (second) leaves inoculated with compatible (Comp.) and incompatible (Incomp.) isolates at 1st (3-a) and 7th (3-b) day after inoculation. Cultivar: Kanto 51, Inocula: Ken 68-17 (Compatible), TH 65-105 (Incompatible)

Table 1. The effect of ample supplies of nitrogenous fertilizer on the peroxidase activity of rice leaves

Treatment	7th leaf	6th leaf
Check	0.301	0.291
Tr-1	0.371	0.441
Tr-2	0.340	0.710

Note: Ammonium sulfate solution was added at full development of 6th leaves, followed by second treatment after 5 days.
Samplings were carried out at full development of 7th leaves.
Tr-1, 2: 188 and 376 mg of ammonium sulfate were amply added respectively.
Numbers show O.D. at 430 nm.

after infection and there were no distinct differences between compatible and incompatible combination except for the starting time of activation and the degree of increase (Fig. 2, 3). No new isozyme which is specific to each combination was observed. Although new isozymes with low activity appeared sometimes after infection, these isozymes also appeared along with the ageing of leaf tissue in intact plant. Ample supplies of N-fertilizer reduced the resistance to blast disease. The peroxidase activities, however, did not correlate with the degree of the resistance. The total activities increased in parallel with the doses of nitrogen fertilizer and highest activity was recorded in most susceptible condition (Table 1).

Discussion

The observation in cell level by using rice-sheath inoculation method indicated a symbiotic relation between host and parasite during early stage of infection in compatible combination of rice cultivar and race of *Pyricularia oryzae* Cav. In incompatible combination, around 70 % of invaded cells died and in compatible cases, on the contrary, 90 % of invaded cells were still alive at 18 hrs after inoculation[32]. The increase of peroxidase activities occured in pararell with such disruption of host cells. The rapid increases of total and isozyme activity of peroxidase were observed in incompatible combinations within 24 hr after inoculation. This increase occured more rapidly in aged rice leaves and correlated with appearance of brown spots on leaf blade. Conversely, no increase of the activites was observed in compatible cases until 3 days after inoculation. Since this observation is in tissue level, the increase of activities might occur more rapidly in cell level. The appearance of new isozyme was reported in rice blast disease[44]. However, no new isozymes which is specific to compatible or incompatible combinations were observed in this experiment. Similar result was reported in powdery mildew of *Trifolium pratense*[46]. Only difference between compatible and incompatible cases is the starting time of the increase.

Since peroxidase plays as a catalyst of various physiological processes, the implication with resistance to various diseases have been much stressed. However, some critical analyses have also been accumulated[1,6,7,8,14,16,17,31,36,40]. Some of them were carried out by using near isogenic lines of wheat carrying *Sr-6* allele[7,8,39,40]. Resistant lines in which peroxidase activities were stimulated by ethylene treatment and/or infection at 20 C, reverted to complete susceptibility after transferring to 26 C, despite the fact that high peroxidase activity were still keeping. Therefore, it was concluded that total peroxidase activity is not causally related to resistance[40]. In our experiment, the activities were rather higher in a compatible combination than a incompatible combination at 5–7 th day after inoculation and 3–7 th day in aged leaves. Furthermore, the highest activity was recorded at the time of rapid enlargement of blast lesion in the compatible combination. In some diseases, the activities were also higher in compatible case[1,6,14,36,43]. These results could indicate that increased enzyme activity is rather a biochemical symptom of disease and tissue destruction[30]. Many reports have suggested these possibilities[3,7~10,15,24,38,40,52,53]. While, increase of the activity unrelated with the general resistance in compatible combination was also observed. Ample supplies of nitrogenous fertilizer caused the reduction of resistance to rice blast disease. The peroxidase activities in this plant is higher than that of the plant which was raised at normal doses of nitrogen fertilizer. The highest activity was recorded in the most susceptible condition[27,28,42]. These results indicated the possibility that the increase of peroxidase activity might not be a limiting factor of the general resistance in compatible combination. Further accumulation of evidence will be needed.

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和 文 摘 要

イネいもち病抵抗性と相関しない Peroxidase の活性増について

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非親和性のイネ品種-いもち病菌菌系の組合せに於て Peroxidase の総活性は接種後24時間以内に急激に増加するのに比べ，親和性の組合せに於ては感染初期では殆んど変化せず，感染後3～5日目にかけて急速に増加し非親和性の場合をはるかに陵駕した。このような経時的変化は age が進んだ下位葉に於て更に急激に起こった。最も高い活性は親和性の組合せで観察され，しかも病斑が最も急激に拡大する時期に高かった。同位酵素の活性増は特定の酵素に限られており，感染により特異的に新しい同位酵素が出現することは無かった。活性増の起こる時期，程度を除けば親和性，非親和性に極端な違いは認められない。N肥多施用はいもち病抵抗性を著しく低下させるが Peroxidase 活性はN肥の施用量に比例して増加し，最も罹病的な状態の時に最も高い活性を示した。以上の結果は Peroxidase の活性増が単なる細胞の崩壊，生理急変に伴う代謝変動であり抵抗性，抵抗力と直接関係していない可能性を示すとも考えられ，今後の検討が必要である。